Does Not Contain TSCA CBI

SUBJECT: Construct Hazard Analysis for TERA R-19-0001

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I. Introduction

EPA has received a TSCA Environmental Release Application (TERA) from Synthetic Genomics, Inc. to test one intergeneric eukaryotic algal strain in open ponds. The subject microorganism of this non-CBI TERA R-19-0001 is the green alga *Parachlorella* sp., strain STR26155. The wild-type parent strain STR00010 strain was obtained off the coast of the Hawaiian island of Oahu. The recipient strain STR00012, which was derived from STR00010 following UV mutagenesis, was engineered to express a "Turbo" green fluorescent protein (GFP) gene. The TurboGFP gene was obtained from Evrogen (Evrogen Joint Stock Company, Moscow, Russia, Catalog #FP511). TurboGFP was developed by Evdokimov et al. (2006) by performing solubility-enhancing optimization of ppluGFP2, the Copepoda GPF originally isolated from the *Pontellina plumata* in the order Calanoida (Shagin et al., 2004). TurboGFP is a highly water soluble, rapidly maturing variant of ppluGFP2 with a brighter fluorescence than ppluGFP2. According to the Genetic Construction Report (Cameron, 2019) this specific TurboGFP gene purchased and used in creation of STR26155 has been codon-optimized for expression in mammalian cells by Evrogen according to the method used by Haas et al. (1996) for optimizing expression of proteins in mammalian systems.

The introduction of the TurboGFP into the recipient strain was done to enable environmental tracking of the subject strain STR21655. Environmental monitoring of the recipient strain STR00012 has been done for 18 months. The genetic engineering involved the use of CRISPR-Cas9, transformation by electroporation, and the use of the loxP-Cre recombinase system for removal of antibiotic resistance genes used in selection of intermediates. Although resistance genes for chloramphenicol and zeocin were used as selection markers, no antibiotic resistance genes remain in the subject microorganism STR26155.

The final strain STR26155 contains the TurboGFP gene, an intergeneric loxP site, and an intrageneric *HpaI* (restriction enzyme) site.

This is the fourth TERA received by the Agency for open pond testing of genetically engineered algae. Previous algae TERAs include R-13-0003 to -0007, R-17-0002, and R-18-0001.

II. Genetic Construction of the Microorganism

Details of the genetic modifications done to arrive at the subject strain STR26155 are presented in the Genetic Construction Report (Cameron, 2019). As stated above, the parental wild-type strain, *Parachlorella sp.,* was isolated from seawater in Hawaii. This strain, designated as STR00010, was subjected to UV mutagenesis. A recipient strain, designated as STR00012, was chosen as it demonstrated higher biomass productivity compared to the wild-type strain. A plasmid (NAS14335) containing the TurboGFP cassette was constructed via Gibson cloning of eight linear DNA fragments, synthetic linkers, and genes, PCR-amplified endogenous regulators, and intermediate plasmids. This plasmid was digested with a restriction enzyme prior to co-transformation into the recipient using a CRISPR Cas9 nuclease ribonucleoprotein (RNP) complex (Cas9 with guide RNA). The plasmid backbone contained a chloramphenicol resistance gene, but this gene as well as several others dropped out of the intermediate strain as the backbone of the plasmid did not have the ability to self-replicate. Sequencing of the subject microorganism confirmed the backbone did not integrate off-target in the genome.

This NAS14335 plasmid was originally assembled so that the Cre recombinase and *ble* genes were in between the two *loxP* sites so that Cre recombinase could cause self-excision of the DNA between the *loxP* sites. The Cre recombinase was then induced in the presence of nitrate which resulted in the removal of the *ble* gene (zeocin resistance) used as a selection marker. The removal of the *ble* gene was verified by zeocin sensitivity.

The site of insertion of the TurboGFP was the RS1 locus. The RS1 site was specifically selected as a neutral region with no nearby coding sequences. The correct insertion site was verified by sequencing.

The intergeneric TurboGFP gene and one *loxP* site (from bacteriophage P1) remains in the subject microorganism STR26155. There is also one intrageneric *HpaI* site remaining in STR26155 from the genetic modifications.

III. Hazards of the Introduced Genetic Material

A. TurboGFP

The subject strain is engineered to express the TurboGFP for monitoring in the environment. As previously stated, TurboGFP is a variant of the ppluGFP2 originally isolated from the copepod *Pontellina plumata* (Shagin et al., 2004). TurboGFP, developed by Evdokimov et al. (2006), has a faster maturing brighter fluorescence that the ppluGFP2 from which it is derived. This specific TurboGFP purchased from Evrogen is a version of the TurboGFP developed by Evdokimov et al. (2006) that has been codon-optimized by Evrogen for expression in mammalian cells following the method of Haas et al. (1996). However, this TurboGFP can be successfully expressed in many other systems.

The green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria,* was the first of a number of fluorescent proteins isolated from ocean dwelling-organisms (Shimomura et al., 1962; Johnson et al., 1962). GFP has since been used as an *in vivo* fluorescent marker in a wide variety of microbial, plant, and animal studies (Shagin et al., 2004; Taghizadeh and Sherley, 2008). This GFP does not require substrates or cofactors (Taghizadeh and Sherley, 2008). Chalfie

et al. (1994) was the first to report the introduction of the GFP in other organisms, both in the bacterium *Escherichia coli* and in the nematode, *Caenorhabditis elegans*. Yang et al. (1996) elucidated the structure of the green fluorescent protein that consists of 238 amino acids in a cylinder shape comprised of 11 strands of β-sheets with an α-helix inside and short helical segments on the ends of the cylinder. They stated that this structure was a new protein fold not previously observed for any other proteins in nature, which they named the β-can. The cyclization of serine-dehydroxytyrosine-glycine within the α-helix coil in the center of the protein is responsible for the fluorescent chromophore (Cody et al., 1993). It is the barrel structure of the protein that protects the chromophore and provides for its extreme stability. In an interview, several researchers speculated that the barrel structure was also responsible for its lack of toxicity in the cells into which it is introduced (Manning, 1997). The extreme stability of the protein has been demonstrated in numerous studies. As summarized by Haseloff (1998), the fluorescence of this protein has been shown to be unaffected by extended treatment with 6 M guanidine HCl, 8 M urea, or 1% sodium dodecyl sulfate. It was unaffected by 2-day treatments with up to 1 mg/ml of the proteases trypsin, chymotrypsin, papain, subtilisin, thermolysin, and pancreatin. The protein has been shown to be stable up to 65 °C in neutral buffer, and in a pH range of 5.5 to 12. Andersen et al. (1998) reported that the wild-type GFP in *E. coli* had a half-life of greater than 24 hrs. In studies with GFP-tagged *Pseudomonas fluorescens*, Lowder et al. (2000) reported that fluorescence was stable during the entire 6-month incubation period when cells were in the viable but non-culturable (VBNC) state. In the same article, the authors reported that when log-phase cells were killed by UV light, the fluorescence of the supernatant surpassed that of the dead cells which indicated that rather than being degraded, the fluorescent protein was released intact from the cells into the surrounding environment.

The extreme stability of wild-type GFP has proven to be disadvantageous in some applications since it can interfere with the ability to monitor metabolic activity or even cell death. In addition, background interference in environmental samples, particularly in soils, occurs frequently. Consequently, numerous variants of the original GFP with different half-lives or with different emission wavelengths have been developed. An enhanced GFP (eGFP) was developed to alleviate the problems encountered with persistence of the wild-type GFP. Numerous derivatives of GFP and other fluorescent genes with different stability and absorbance/ excitation peaks have been created for various applications (Mankin and Thomas, 2001; Zhang et al., 2002). In addition, other fluorescent proteins derived from a group of reef corals belonging to the class Anthozoa such as DsRed2, DsRed Express, DsRed-monomer, AsRed2, HcRed1, AmCyan, ZsGreen, ZsYellow, and p-Timer, and AcGFP1 are now commercially available (from Clontech). Various other fluorescent proteins have been isolated from other ocean-dwelling organisms. As previously mentioned, TurboGFP is a variant of the CopGFP originally isolated from the copepod *Pontellina plumata*. The excitation/emission max of this TurboGFP is 482/502 nm (Cameron, 2019).

There are also reports in the literature suggesting that GFP and some derivatives may present problems in certain constructs in animals and plants (Liu et al., 1999). Aggregation toxicity with GFP has also been reported with a C-terminal addition of a short peptide in *Caenorhabditis elegans* (Link et al., 2005). In studies using viruses marked with eGFP in retinal cells of rabbits, there was one instance where abnormal morphology was encountered, and the authors speculated that it may have been an immune response rather than toxicity of the eGFP itself (Rex et al., 2005). In most of these viral studies, eGFP up to a several hundred micromolar

concentration was not detrimental to retinal cells. In plant cells, Haseloff et al. (1997) reported that GFP could be toxic under high light conditions with high protein expression. In addition, Taghizadeh and Sherley (2008) found that GFP was toxic in rat hepatic adult stem cells, however, yellow fluorescent protein (YFP) or cyan fluorescent protein (CFP) worked well as stable marker genes in these cells.

There is one study in the literature assessing the toxicity of GFP fed to rats as pure protein and in a diet consisting of transgenic canola expressing GFP (Richards et al., 2003). The authors reported that oral administration of 1.0 mg of purified GFP/day for 26 days was not toxic to male rats. However, there was a slight, but significant, decline in weight gain in rats fed the GFP canola diet although this was the only parameter affected. The authors reported that GFP was readily degraded in a simulated gastric digestibility study, however, GFP fluorescence was observed in feces of rats fed purified GFP. The authors recommended that their preliminary conclusion that "GFP represents a minimal risk in the food supply" be further investigated in long-term feeding studies. A database search for similarity to known food allergens found only a four amino acid sequence match which was not considered significant which suggested the absence of allergenic epitopes (Richards et al., 2003).

The biological function of these fluorescent proteins in the organisms from which they were isolated is unknown. Long ago, it was hypothesized that fluorescent proteins may serve as protection from strong solar radiation for organisms in shallow waters (Kawaguti, 1944, as cited by Matz et al., 1999). A more recent article (Salih et al., 2000) reported that fluorescent pigments serve a photoprotective function in corals. For organisms in deep waters where light is mostly blue because it is depleted of low-energy components, it has been suggested that the function of the fluorescent proteins may be to provide longer wavelengths than the blue light which may be better for photosynthesis by algal endosymbionts (Schlichter et al., 1994). Shagin et al. (2004) stated that thus far, there seems to more evidence to support photoprotection of endosymbiotic algae, however, the function of these fluorescent proteins is still controversial.

Although there have been studies reporting problems with using GFP in some cells, the TurboGFP used in this *Parachlorella* sp. apparently does not pose problems. According to Evrogen's website (http://evrogen.com/products/TurboGFP/TurboGFP_Detailed_description.shtml), no cytotoxic effects or visible protein aggregation are observed with TurboGFP. TurboGFP was developed by Evdokimov et al. (2006) to overcome some of the problems associated with Copepoda GFPs. These problems include (1) Copepoda GFPs are prone to form aggregates and (2) overexpression of Copepoda GFPs in mammalian cells can lead to the formation of microcrystals that can rupture cell membranes. Therefore, Evdokimov et al. (2006) developed TurboGFP which is a highly soluble, rapidly maturing variant derived from ppluGFP2 (Shagin et al., 2004) (GenBank accession number AY268072). The authors reported that its crystal structure is a β-barrel fold that exists as a dimer in solution at concentrations at least up to 5 mg/ml, and forms tetramers in the crystal form. According to the authors, the TurboGFP chromophore is similar to that of other GFPs that have an abundance of buried charged side chains typical of internalized catalytic centres. It is the residues that contribute functional groups that vary with the different GFPS in the GFP family, but the chromophore-relative positions of important functional groups are conserved. Evdokimov et al. (2006) succeeded in expressing high levels of TurboGFP in bacteria, yeast, and mammalian cells.

Evdokimov et al. (2006) also explained the rapid development of the fluorescence. A unique feature of TurboGFP is a water-filled pore leading from the outside of the protein barrel to Y5B of the chromophore. The authors suggested that this pore facilitates oxygen conveyance to the premature chromophore that speeds up the maturation of the fluorescence.

The TurboGFP apparently functions well as a stable marker gene in this *Parachlorella* algal strain STR26155.

B. loxP site

A single loxP site remains in the genome of the subject microorganism STR26155. The loxP site is from bacteriophage P1 (as is the Cre recombinase enzyme). It is a 34 bp DNA sequence as follows (where N means that the base may change):

13bp 8bp 13bp

ATAACTTCGTATA - NNNTANNN -TATACGAAGTTAT

The two end 13 bp sequences are palindromic. The 8 bp internal sequence is not. In genetic engineering, a pair of loxP sites is used. If the two loxP sites are in the same orientation, the floxed sequence (sequence flanked by two loxP sites) is excised. This well-known system for excision of DNA sequences in between the two loxP sites was used to remove the zeocin resistance gene (*ble*) in creation of the subject strain STR26155. The Cre recombinase and one loxP site were also lost.

This one loxP sequence remaining in STR26155 does not pose any hazards.

C. Intrageneric *HpaI* site

In addition to the two intergeneric sequences, the subject strain STR26155 also contains an intrageneric *HpaI* site. This is merely a site with a sequence of 5' …GTT | AAC…3' or 3' …CAA | TTG… 5' where restriction enzyme *HpaI* will cut.

The presence of this site in STR26155 does not pose any hazards.

IV. Potential for Gene Instability and Transfer

With environmental introduction of genetically engineered microorganisms, the potential for horizontal gene transfer of introduced genes into other microorganism in the environment warrants consideration. Horizontal gene transfer among bacteria is widespread and is responsible for acquisition of a myriad of traits in bacteria such as antibiotic resistance, xenobiotic degradation pathways, and even pathogenesis. Not nearly as much is known regarding horizontal gene transfer in eukaryotes. It has been thought that the barriers to horizontal gene transfer in bacteria are even worse in eukaryotic organisms because of the complexities in their transcription and translation mechanisms (Raymond and Blankenship, 2003). However, from evolutionary analyses, horizontal gene transfer in eukaryotes is known to have occurred. For example, in evolutionary times, it was a primary endosymbiotic event of a cyanobacterium being engulfed that gave rise to the photosynthetic plastid in the common

ancestor of the Plantae, such as red and green algae and higher plants (Chan et al., 2012). Likewise, the mitochondria arose from the endosymbiosis and subsequent genetic integration of an alpha-proteobacterium (Keeling and Palmer, 2008). In addition, investigations of the *Chlorella* genome, specifically *Chlorella variabilis*, suggest the ability for *Chlorella* to produce chitinous cell walls as a result of genetic material uptake from algal viruses, prokaryotes, and fungi (Blanc et al., 2010). Eckardt et al. (2010) hypothesized that the *Chlorella* chitin metabolism genes could have been acquired via horizontal gene transfer from viruses. There are other episodes of lateral gene transfer in eukaryotes, such as the phagocytosis by the sea slug *Elysia chlorotica* of the alga *Vaucheria litorea.* The photosynthetic sea slug maintains the algal plastids which continue to photosynthesize for months within the slug (Rumpho et al., 2008).

Very little is known about horizontal gene transfer from one algal species to another. A search of the literature on horizontal gene transfer in *Parachlorella* did not reveal any studies specifically on horizontal gene transfer in *Parachlorella.* However, there is also evolutionary evidence for horizontal gene transfer in algae. Archibald et al. (2003) found that of the 78 plastid-targeted proteins in the chlorarachniophyte alga *Bigelowiella natans*, approximately 21% of them had probably been acquired from other organisms including streptophyte algae, red algae (or algae with red algal endosymbionts), and bacteria. However, in the green alga *Chlamydomonas reinhardtii,* the homologous genes did not show any evidence of lateral gene transfer. It was suggested that this may be because this green alga is solely autotrophic whereas the *Bigelowiella* is both photosynthetic and phagotrophic. Another instance of potential lateral gene transfer having occurred in algae is the work presented by Raymond and Kim (2012). They found the presence of ice-binding proteins in sea ice diatoms that apparently were essential for their survival in the ice. These protein genes were completely incongruent with algal phylogeny, and the best matches were all bacterial genes. Like bacterial genes, they did not contain introns. There is one example of horizontal gene transfer from an alga to its DNA virus. By phylogenetic analysis, Monier et al. (2013) demonstrated that the transfer of an entire metabolic pathway, consisting of seven genes involved in the sphingolipid biosynthesis, from the eukaryotic alga *Emiliania huxleyi* and its large DNA virus known as EhV had occurred. Hunsperger et al. (2015) reported the conserved presence of the light-dependent protochlorophyllide oxidoreductases (POR) in four different algal taxa (dinoflagellates, chlorarachniophytes, stramenopiles, and haptophytes). The study concluded that the duplicates of stramenopiles and haptophytes *por* genes are a result of horizontal gene transfer from a Prasinophyte alga. A recent study revealed a shared ancestry between the Pedinomonadales and Chlorellales algae after sequencing the chloroplast genome of *Pedinomonas minor* (Pedinomonadales)*,* two trebouxiophyceans, *Parachlorella kessleri* (Chlorallaceae) and *Oocystis solitaria* (Oocystaceae), and comparing the sequences to the chloroplast genome of *Chlorella vulgaris* (Turmel et al., 2015).

There is no information in the literature on horizontal gene transfer specifically with *Parachlorella* nor on the closely related genus *Chlorella*. The intergeneric TurboGFP gene is stably integrated into the chromosome which lessens the likelihood of horizontal gene transfer. It is unlikely that the TurboGFP gene would be transferred to and expressed in other green algae as it does not provide for any selective advantage in the environment. Even if horizontal gene transfer was to occur, the TurboGFP poses low hazards. Although from an evolutionary perspective there is evidence that horizontal gene transfer has occurred in green algae, there are no studies that demonstrate horizontal gene transfer with *Parachlorella,* or the closely related genus *Chlorella,* and other algae.

V. Conclusions

Parachlorella sp. is not known to produce any toxins that might be harmful to humans, animals, or plants. Although resistance genes to chloramphenicol and zeocin were used in the development of the subject strain STR26155, they are not present in this final submission strain. The genetic modifications made to the recipient microorganism involved introducing the TurboGFP. This modification is not expected to introduce any other phenotypic change in the recipient microorganism and does not impart or enhance any harmful traits beyond what may be present in the recipient strain. The proposed field test of STR26155 poses low concern for humans and the environment as the genetic modification of introducing TurboGFP also poses low hazards.

References:

Andersen, J.B., C. Sternberg, L.K. Poulsen, S.P. Bjorn, M. Givskov, and S. Molin. 1998. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl. Environ. Microbiol. 64:2240 -6.

Archibald, J.M., M.B. Rogers, M. Toop, K.-I. Ishida, and P.J. Keeling. 2003. Lateral gene transfer and the evolution of plastid-targeted proteins in the secondary plastid-containing alga *Bigelowiella natans.* Proc. Natl. Acad. Sci. 100:7678-83.

Cameron, S. 2019. Genetic Construction Report for R-19-0001. Office of Pollution Prevention and Toxics. Environmental Protection Agency. Washington, DC.

Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward, D.C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. Sci. 263:802-5.

Chan, C.X., D. Bhattacharya, and A. Reyes-Prieto. 2012. Endosymbiotic and horizontal gene transfer in microbial eukaryotes. Impacts on the tree of life. Mobile Genetic Elements. 2:101-5.

Cody, C.W., D.C. Prasher, W.M. Westler, F.G. Prendergast, and W.W. Ward. 1993. Chemical structure of the hexapeptide chromophore of the *Aequorea* green fluorescent protein. Biochem. 32:1212-18.

Eckardt, N.A. 2010. The *Chlorella* genome: Big surprises from a small package. Plant Cell 22: 2924.

Evdokimov, A.G., M.E. Pokross, N.S. Egorov, A.G. Zaraisky, I.V. Yampolsky, E.M. Merzlyak, A.N. Shkoporov, I.S. Sander, K.A. Lukyanov, and D.M. Chudakov. 2006. Structural basis for the fast maturation of Arthropoda green fluorescent protein. EMBO Reports 7:1006-1012.

Haas, J., Park, E.C., and B. Seed. 1996. Codon usage limitation in the expression of HIV-1 envelope glycoprotein. Curr. Biol. 6(3): 315-324.

Haseloff, J., K.R. Siemering, D.C. Prasher, and S. Hodge. 1997. Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis*

plants brightly. Proc. Natl. Acad. Sci. U.S.A. 94:2122-7.

Haseloff, J. 1998. Imaging green fluorescent protein in transgenic plants. *In:* R. Rissuto and C. Fasolato (eds.) Imaging Living Cells, pp. 362-394, Springer Nature, Switzerland. (https://data.plantsci.cam.ac.uk/Haseloff/resources/LabPapers/Haseloff98.pdf).

Hunsperger, H.M., T. Randhawa and R.A. Cattolico. 2015. Extensive horizontal gene transfer, duplication, and loss of chlorophyll synthesis genes in the algae. BMC Evolutionary Biology 15:16. DOI 10.1186/s12862-015-0286-4.

Johnson, F.H., L.C. Gershman, J.R. Waters, G.T. Reynolds, Y. Saiga, and O. Shimomura. 1962. Quantum efficiency of Cypridina luminescence, with a note that of Aequorea. J. Cell. Comp. Physiol. 60:85-104.

Kawaguti, S. 1944. On the physiology of reef corals. VI. Study of the pigments. Palso Trop. Biol. Sin. Stud. 2:617-674.

Keeling, P.J., and J.D. Palmer. 2008. Horizontal gene transfer in eukaryotic evolution. Nature Rev. 9:605-18.

Link, C.D., V. Fonte, B. Hiester, J. Yerg, J. Ferguson, S. Csontos, M.A. Silverman, and G.H. Stein. 2006. Conversion of green fluorescent protein into a toxic, aggregation-prone protein by C-terminal addition of a short peptide. J. Biological Chem. 281:1808-16.

Liu, H.S., M.S. Jan, C.K. Chou, P.H. Chen, and N.J. Ke. 1999. Is green fluorescent protein toxic to the living cell? Biochem. Biophys. Res. Commun. Jul 14 ; 260(3):712-7.

Lowder, M., A. Unge, N. Maraha, J.K. Jansson, J. Swiggett, and J.D. Oliver. 2000. Effect of starvation and the viable-but-nonculturable state on green fluorescent protein (gfp) fluorescence in gfp-tagged *Pseudomonas fluorescens* A506. Appl. Environ. Microbiol. 66:3160-3165.

Mankin, S.L. and W.F. Thomas. 2001. New green fluorescent protein genes for plant transformation: Intron-containing, ER-localized, and soluble-modified. Plant Molecular Biology Reporter 19:13-26.

Manning, E. 1997. Glow fish: An unusual glowing molecule from jellyfish is helping to illuminate cellular events. Bioscience 47(3):135-138.

Matz, M.V., A.F. Fradkov, Y.A. Labas, A.P. Savitsky, A.G. Zaraisky, M.L. Marketov, and S.A. Lukyanov. 1999. Fluorescent proteins from nonbioluminescent Anthozoa species. Nature Biotech. 17:969-973.

Monier, A., A. Pagarete, C. de Vargas, M.J. Allen, B. Read, J.-M. Claverie, and H. Ogata. 2009. Horizontal gene transfer of an entire metabolic pathway between a eukaryotic alga and its DNA virus. Genome Research 19:1441-9.

Raymond, J.A. and R.E. Blankenship. 2003. Horizontal gene transfer in eukaryotic algal evolution. Proc. Natl. Acad. Sci. 100:7419-20.

Raymond, J.A. and H.J. Kim. 2012. Possible role of horizontal gene transfer in the colonization of sea ice by algae. PLoS ONE. 7(5):e35968.

Rex, T.S., J.A. Peet, E.M. Surace, P.D. Calvert, S.S. Nikonov, A.L. Lyubarsky, E. Bendo, T. Jughes, E.N. Pugh, and J. Bennett. 2005. The distribution, concentration, and toxicity of enhanced green fluorescent protein in retinal cells after genomic or somatic (virus-mediated) gene transfer. Molecular Vision11:1236-45.

Richards, H.A., C.-T. Han, R.G. Hopkins, M.L. Failla, W.W. Ward, and C.N. Stewart, Jr. 2003. Safety assessment of green fluorescent protein orally administered to weaned rats. J. Nutr. 133:1909-12.

Rumpho, M.E., J.M. Worfula, J. Leeb, K. Kannana, M.S. Tylerc, D.Bhattacharyad, A. Moustafad, and J.R. Manharte. 2008. Horizontal gene transfer of the algal nuclear gene *psbO* to the photosynthetic sea slug *Elysia chlorotica*. Proc. Natl. Acad. Sci. 105:17867-71.

Salih, A., A. Larkum, G. Cox, M. Kuhl, O. Hoegh-Guldberg. 2000. Fluorescent pigments in corals are photoprotective. Nature 408:850-3.

Schlichter, D., U. Maier, and H.W. Fricke. 1994. Improvement of photosynthesis in zooanthallate corals by autofluorescent chromatophores. Oecologia 99:124-131.

Shagin, D.A., E.V. Barsova, Y.G. Yanushevich, A.F. Fradkov, K.A. Lukyanov, Y.A., Labas, T.N. Semenova, J.A. Ugalde, A. Meyers, J. Nunez, E.A. Widder, S.A., Lukyanov, and M.V. Matz. 2004. GFP-like proteins as ubiquitous metazoan superfamily: Evolution of functional features and structural complexity. Molec. Biology Evolution. 21:841-850.

Shimomura, O., F.H. Johnson, and Y. Saiga. 1962. Extraction, purification, and properties of Aequorin, a bioluminescent protein from luminous Hydromedusan. J. Cell. Comp. Physiol. 59:223-239.

Taghizadeh, R.R. and J.L. Sherley. 2008. CFP and YFP, but Not GFP, Provide Stable Fluorescent Marking of Rat Hepatic Adult Stem Cells. J. Biomed. Biotechnol. Volume 2008, Article ID 453590, 9 pages, doi:10.1155/2008/453590.

Turmel, M., C. Otis, and C. Lemieux. 2009. The chloroplast genomes of the green algae *Pedinomonas minor, Parachlorella kessleri,* and *Oocystis solitaria* reveal a shared ancestry between the Pedinomonadales and Chlorellales. Mol. Biol. Evol.26:2317-2331.

Yang, F., L.G. Moss, and G.N. Phillips. 1996. The molecular structure of green fluorescent protein. Nature Biotechnol. 14:1246-1251.

Zhang, J., R.E. Campbell, A.Y. Ting, and R.Y. Tsien. 2002. Creating new fluorescent probes for cell biology. www.nature.com/reviews/molcellbio 3:906-918.